Science of the Total Environment 625 (2018) 782–791

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Shifts in soil bacterial and archaeal communities during freeze-thaw cycles in a seasonal frozen marsh, Northeast China

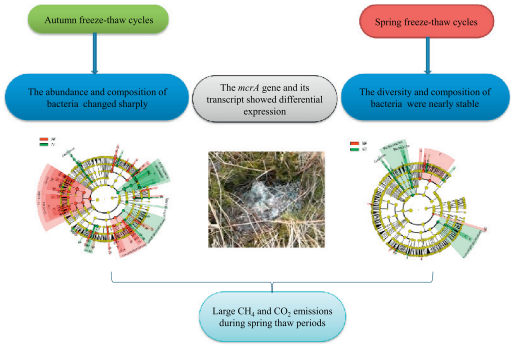
Jiusheng Ren [a,b], Changchun Song [a,*], Aixin Hou [c], Yanyu Song [a], Xiaoyan Zhu [d], Grace Ann Cagle [c]

[a] Key Laboratory of Wetland Ecology and Environment, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Changchun 130102, PR China
[b] University of Chinese Academy of Sciences, Beijing 100049, PR China
[c] Department of Environmental Sciences, College of the Coast and Environment, Louisiana State University, Baton Rouge, LA 70803, USA
[d] Key Laboratory of Songliao Aquatic Environment, Ministry of Education, Jilin Jianzhu University, Xincheng Street, District 5088, Changchun 130118, PR China

HIGHLIGHTS

- Spring and autumn FTCs conversely affect quantities of bacteria and methanogens.
- Bacterial diversity and abundance changed sharply upon autumn FTCs.
- Temperature and substrates mainly regulate bacterial abundance and composition.
- Spring large CH_4 emissions mainly came from autumn FTCs in seasonal frozen marsh.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 6 September 2017
Received in revised form 19 December 2017
Accepted 27 December 2017
Available online 3 January 2018

Editor: Solo-Gabriele

Keywords:
Freeze-thaw cycles
Methane
Illumina MiSeq
qPCR
16S rRNA gene
mcrA

ABSTRACT

Diurnal freeze-thaw cycles (FTCs) occur in the spring and autumn in boreal wetlands as soil temperatures rise above freezing during the day and fall below freezing at night. A surge in methane emissions from these systems is frequently documented during spring FTCs, accounting for a large portion of annual emissions. In boreal wetlands, methane is produced as a result of syntrophic microbial processes, mediated by a consortium of fermenting bacteria and methanogenic archaea. Further research is needed to determine whether FTCs enhance microbial metabolism related to methane production through the cryogenic decomposition of soil organic matter. Previous studies observed large methane emissions during the spring thawed period in the Sanjiang seasonal frozen marsh of Northeast China. To investigate how FTCs impact the soil microbial community and methanogen abundance and activity, we collected soil cores from the Sanjiang marsh during the FTCs of autumn 2014 and spring 2015. Methanogens were investigated based on expression level of the methyl coenzyme reductase (*mcrA*) gene, and soil bacterial and archaeal community structures were assessed by 16S rRNA gene sequencing. The results show that a decrease in bacteria and methanogens followed autumns FTCs, whereas an increase in bacteria and methanogens was observed following spring FTCs. The bacterial community structure, including *Firmicutes* and certain *Deltaproteobacteria*, was changed following autumn FTCs. Temperature and substrate were the primary factors regulating the abundance and composition of the microbial communities during autumn FTCs, whereas no factors significantly contributing to spring FTCs were identified. Acetoclastic methanogens from order *Methanosarcinales* were the dominant group at the beginning and end of both the autumn and spring

* Corresponding author at: Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, 4888 Shengbei Street, Changchun 130102, PR China.
E-mail address: songcc@iga.ac.cn (C. Song).

https://doi.org/10.1016/j.scitotenv.2017.12.309

FTCs. Active methanogens were significantly more abundant during the diurnal thawed period, indicating that the increasing number of FTCs predicted to occur with global climate change could potentially promote CH_4 emissions in seasonal frozen marshes.

1. Introduction

Freeze-thaw cycles (FTCs) are a common phenomenon in soils of temperate and cold climates. It includes two physical process cycles: freezing and thawing, which can be divided into annual FTCs and multiple diurnal FTCs. The latter has been shown to play a significant role in greenhouse emissions in boreal wetlands (Wei and Wang, 2017; Yu et al., 2007). FTCs can promote the emission of methane (CH_4), a significant greenhouse gas possessing a radiative force 25 times greater than that of carbon dioxide (CO_2) (Change, 2014), and the frequency of diurnal FTCs is expected to increase under climate warming scenarios (Change, 2014; Wang et al., 2017a.

Large CH_4 emissions from boreal wetlands during spring FTCs have received increasing attention in recent years, and they appear to be a significant fraction of the annual CH_4 budget (Song et al., 2012; Tokida et al., 2007). This phenomenon has also been repeatedly observed in arctic wetlands, mires, and tundra, which accounted for 25%, 11% and 6% of the annual budget, respectively (Friborg et al., 1997; Hargreaves et al., 2001; Raz-Yaseef et al., 2017). However, the contribution of CH_4 emissions to the annual flux during FTCs is unclear. Some research found that CO_2 and CH_4 fluxes emitted during autumn and spring FTCs (Liptzin et al., 2009; Mastepanov et al., 2008; Tagesson et al., 2012; Wille et al., 2008), while the other studies found both CO_2 and CH_4 emissions have been observed during winter rather than autumn and spring FTCs in arctic tundra and grassland (Y.H. Wang et al., 2014; Zona et al., 2016). In wetland soils, methanogenesis is driven by a complex community of fermenting bacteria, syntrophic bacteria, and methanogenic archaea (Cicerone and Oremland, 1988). CH_4 release is the direct net result of methanogenesis and CH_4 oxidation processes. However, the microbial dynamics present during large spring CH_4 emissions associated with FTCs have not been well examined.

FTCs, a type of abiotic stress, can create anaerobic environment and increase substrate availability by physically destroying soil aggregates and soil microorganisms (Hentschel et al., 2008; Oztas and Fayetorbay, 2003; Yu et al., 2011). FTCs may also induce structural changes in soil microbial communities that regulate enzyme activity (Kumar et al., 2013; Sharma et al., 2006). However, the strength of the FTCs' effects is dependent upon the ecosystem in which they occur. For example, subtle effects on soil bacteria were observed during FTCs in grassland soil (Feng et al., 2007; Grogan et al., 2004), whereas FTCs significantly reduced soil microbial abundance and activity in arctic-alpine soil and arctic mud flat (Larsen et al., 2002; Sawicka et al., 2010).

Because FTCs experiments have primarily been conducted in the laboratory, realistic field conditions and actual shifts in soil microorganisms cannot be fully replicated (Henry, 2007). Additionally, previous FTCs experiments considered only either autumn FTCs (Groffman et al., 2006; Neilson et al., 2001; Teepe et al., 2001) or spring FTCs (Holst et al., 2008). The responses of soil microorganisms to FTCs have been studied in steppes and forests. But to our knowledge, the in situ process has not been investigated in boreal wetlands (Urakawa et al., 2014; Wang et al., 2017b).

The objective of the present study was to determine how autumn and spring FTCs influence the in situ soil microbial community in a seasonal frozen marsh, located in Sanjiang of Northeast China. To investigate the impact of FTCs on total bacterial abundance, methanogen activity and community structure, we utilized a combined approach of quantitative PCR and 16S rRNA gene sequencing. We made the following hypotheses: i) Spring and autumn FTCs have contrasting effects on the abundance of bacteria and methanogens. We expected that microbial abundance and activity would be less at the start of spring FTCs than at the start of autumnal FTCs due to limited growth during the spring frozen period, compared to the summer growing season leading up to autumnal FTCs. ii) Multiple FTCs will decrease the diversity of bacterial and archaeal communities in both spring and autumn. iii) Large spring CH_4 mainly come from the entire autumn FTCs period due to increased anaerobic conditions and substrate availability for methanogens.

2. Materials and methods

2.1. Site description and soil sampling

The sampling site was located at the Sanjiang Wetland Experimental Station, Chinese Academy of Sciences (47°35′N, 133°31′E). The Sanjiang Plain in Northeast China has an area of 10.89×10^4 km^2, and belongs to the seasonal frozen region (Song et al., 2003). The site experiences a mean annual air temperature of 1.9 °C. The coldest month is January, with a mean temperature of −19.8 °C, and the warmest month is July, with a mean temperature of 21.6 °C. The growing season normally lasted from early May to late September. *Carex lasiocarpa* is the dominant type of vegetation, and the mean water depth is 40 to 50 cm at the sampling site.

Multiple diurnal freezing and thawing periods occurs along the soil profile in this seasonal frozen marsh, and an entire freeze-thaw period includes autumn FTCs of one year and spring FTCs of the next year. In seasonal frozen areas, autumn initially freezes in the surface and gradually freezes in the deeper layer, while spring thawing starts in both the surface and subsoil and moves toward the middle. Based on soil temperatures in the 0–5 cm layer, we collected soil samples on four different dates representing the first frozen phase and the last thawed phase of each season. These occurred on December 15th and 31st of 2014, and April 16th and 19th of 2015, respectively. On each sampling date, we randomly selected four plots in an area wherein large CH_4 emissions were observed by Song et al. (2012). For each plot (a circle with a 1 m diameter), we sampled four soil cores using a sterile cylindrical stainless steel soil sampler with a 10-cm diameter. The cores were comprised of a brown and fibrous root layer, a spongy peat layer, and a pale yellow and sticky gley soil layer. Peat soil layers from 0–5 cm and 5–10 cm were collected, and the corresponding layers were mixed to produce a composite soil sample for each plot, resulting in a total of 32 composite samples from the four sampling dates. Sub-samples for DNA and RNA analysis were stored in a Dewar filled with liquid nitrogen. The remaining soil samples were kept in a sterile insulated can with ice bags for soil physicochemical analysis.

2.2. CH_4 and CO_2 flux measurements

Gas flux measurements were conducted by applying static dark chambers and gas chromatography techniques (Agilent 4890D) with a flame ionization detector (FID). The detailed method for placement of the static dark chamber and gas flux measurements were described in our previous work (Song et al., 2009; Song et al., 2012).

2.3. Physicochemical analysis

Soil and air temperatures were acquired from an in situ meteorological station. Soil moisture was measured by oven-drying at 105 °C for

24 h. Soil pH was measured in soil slurry at a soil-to-water ratio of 1:5 using a calibrated pH meter with a combination electrode (PHS-25, Shanghai, China). Dissolved organic carbon (DOC) was measured with a Multi N/C 2100 analyzer (Analytik Jena AG, Germany) as described by (Ghani et al., 2003).

2.4. Nucleic acid extraction and cDNA synthesis

Total DNA was extracted in triplicate from each composite soil sample using an MP Fast DNA® SPIN Kit for Soil and a FastPrep® instrument (MP Biomedicals, California, USA) according to the manufacturer's instructions. The extracted DNA was quantified using a NanoDrop® ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc., USA), and stored at −20 °C or −80 °C prior to further analysis. Total RNA was extracted from each composite soil sample using a Total RNA PowerSoil® Isolation Kit (MO BIO, USA) following the manufacturer's instructions, and the extracted RNA quantified using the NanoDrop® ND-2000 spectrophotometer. RNA was reverse transcribed using random primers and oligo (dT) primers from the ReverTra Ace qPCR RT Kit (TOYOBO, Japan) following the manufacturer's instructions. Reverse-transcribed cDNA was stored at −80 °C until further analysis.

2.5. qPCR assay

A total of 32 composite soil samples from different seasonal FTCs were assessed by qPCR. The abundances of bacteria and methanogens were determined by qPCR using the bacteria-targeted primers 338F (5′-CCTACGGGAGGCAGCAG-3′) and 518R (5′-ATTACCGCGGCTGCTGG-3′) and the mcrA-targeted primers mlas (5′-GGTGGTGTMGGDTTCA CMCARTA-3′) and mcrA-rev (5′-CGTTCATBGCGTTVGGRTAGT-3′), respectively (Steinberg and Regan, 2009). The reaction mixture contained 2 × SYBR Green master mix (Takara, China), 250 nM of each primer, diluted extracted DNA, and double-distilled H_2O in a final volume of 20 μl. PCR was performed on an ABI Step One Plus machine. The bacterial thermal program consisted of an initial denaturation step at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The thermal program for DNA and cDNA amplification of *mcrA* gene consisted of an initial denaturation step at 95 °C for 3.5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 30 s. Fluorescence data were acquired at 83 °C during an additional temperature step (15 s at a temperature above the melting point of the primer dimers). The efficiencies of *mcrA* and 16S rRNA were 93% and 104%, respectively.

Serial dilutions of linearized plasmid DNA containing the targeted genes were used to construct standard curves. PCR products were pooled, purified by agarose gel electrophoresis and gel-purified with an Axygen Extraction Kit (Axygen, USA). The pUCm-T Vector System Kit (Sangon, China) was used to ligate PCR amplicons following the manufacturer's instructions. The gene ligation mix was transformed via heat-shock exposure to competent *Escherichia coli* DH5α cells. Transformants were selected by performing blue-white colony screening and colony PCR assays employing the M13 forward and reverse primers (Invitrogen, USA). PCR products of the appropriate size were sequenced by Sangon (Shanghai, China).

2.6. Illumina MiSeq sequencing process

Surface layer (0–5 cm) samples obtained from the seasonal FTCs were assessed by sequencing of the 16S rRNA gene. The V4 region of the 16S rRNA gene was amplified with the primer pair 515F (5′-GTGC CAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) combined with Illumina adapter sequences, a pad, and a two-base linker, along with barcodes on the reverse primers (Caporaso et al., 2012). Sample libraries were generated from purified PCR products. A MiSeq 300 Cycles Kit was used to sequence 2 × 150 bp paired-end reads on a MiSeq machine (Illumina, San Diego, CA, USA).

2.7. Bioinformatic analyses

Raw barcoded sequence reads were subjected to quality controls and clustered into 97% similarity operational taxonomic units (OTUs). Low quality sequences were removed if 1) they were not a perfect match to barcode sequences, 2) the quality score was not higher than 20 over a 5-bp window size, or 3) they were short reads < 150 bp (Kong, 2011). Forward and reverse reads with overlaps of at least 10 bp and lower than 0.05 mismatches were joined using FLASH (Magoc and Salzberg, 2011). After ambiguous bases containing "N" were trimmed, joined sequences with lengths between 240 and 260 bp were subjected to chimera removal by U-Chime (Edgar et al., 2011). Lastly, OTU clustering was performed using UCLUST at a similarity level of 97% (Edgar, 2010), and taxonomic groups were assigned using RDP Classifier (Wang et al., 2007) with a minimal 50% confidence estimate. Subsequent analyses were performed in R (R Core Team, 2013). Singletons were removed for downstream analyses and samples were rarefied at 10,000 sequences per sample. Dissimilarity tests were based on the Bray-Curtis dissimilarity index using analysis of similarities (ANOSIM) (Clarke, 1993) with the VEGAN package (v.2.0-3) (Oksanen et al., 2012). Principal coordinate analysis (PCoA) was based on the weighted UniFrac distance matrix employing the classical multidimensional scaling method (Cox and Cox, 2001). Metastats (http://metastats.cbcb.umd.edu/) and R (v3.0.1) were used to determine which taxonomic groups differed significantly during freeze-thaw period. Differentially abundant soil microorganisms were identified by linear discriminant analysis (LDA) effect size (LEfSe). The significance threshold of the alpha parameter for the Kruskal-Wallis (KW) test and the Wilcoxon test among classes and subclasses was set to 0.05, and the cut-off logarithmic LDA score was 2.0. These analyses were performed through the Galaxy server (Segata et al., 2011). DNA sequences were deposited under NCBI SRA accession SRP076424.

3. Results

3.1. CH_4 and CO_2 fluxes in seasonal frozen marsh

Large emissions of both CH_4 and CO_2 were observed during the spring thaw period, which began close to April 20th and lasted approximately four days (Fig. 1). The hourly emission rate of CH_4 was as high as 42.5 g $C/m^2/h^1$ via bubbling emission nearly three orders of magnitude greater than the average flux rate during the growing season of 2014 (Table S1). Compared with growing season of 2014, a larger amount and percentage of CH_4 was emitted during the spring thaw of 2015 (Table S1).

3.2. Soil characteristics during FTCs and FTCs patterns

During autumn and spring FTCs, soil temperature fluctuated between −0.2 and +0.6 in the 0–5 cm layer (Figs. S2 and S3). DOC concentrations in the 0–5 cm layer were significantly higher than those in the 5–10 cm layer ($P < 0.05$), reaching a maximum value of 707.51 mg/kg after multiple autumn FTCs (Table 1). Soil pH during autumn FTCs was lower than that during spring FTCs. Autumn FTCs initially commenced in 0–5 cm layer in early December 2014, then extended to other soil layers, lasting for approximately 72 days (Fig. S2). During spring FTCs, soil FTCs commenced at both the 0–5 cm and 15–20 cm layers simultaneously (Fig. S3). The soil profile completely thawed from April 20th to 24th, which coincided with the time period of the observed CH_4 and CO_2 emission pulses (Fig. 1). Meanwhile, the soil water content increased significantly in the 0–5 cm layer following spring FTCs (Table 1).

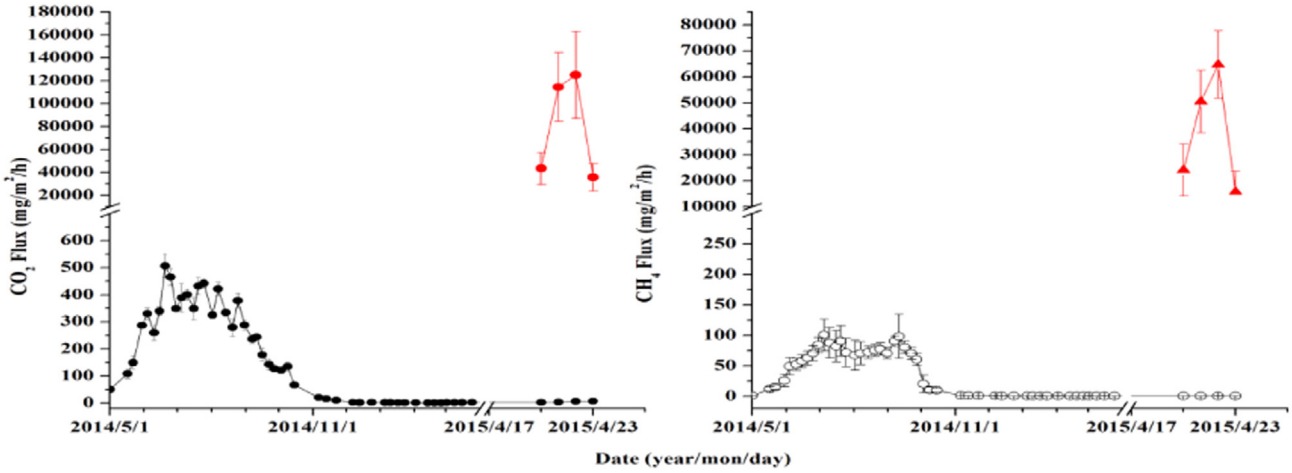


Fig. 1. Observed daily CO_2 and CH_4 fluxes in the Sanjiang seasonal frozen marsh (47.6N,133.5E), Northeast China (the red circles and triangle indicate captured CO_2 and CH_4 pulses, respectively, during the spring thaw period of 2015; the black and white circles indicate the normal CO_2 and CH_4 emissions, respectively, from May 2014 to Apr 2015).

3.3. Gene and transcript abundance during FTCs

Autumn FTCs decreased bacterial and methanogen gene abundances, but increased the *mcrA* transcripts abundance in the 0–5 cm layer (Fig. 2). The 16S rRNA gene abundance decreased by 25%, and the *mcrA* abundance decreased 50% from the first frozen period to the last thawed period. However, the abundance of *mcrA* transcripts was significantly higher during the last autumn thawed period than that during the first frozen period, increasing approximately 2-fold after the FTCs. In the 5–10 cm layer, 16S rRNA gene, *mcrA* gene and *mcrA* transcript abundance did not change significantly following the autumn FTCs.

During the last thaw following spring FTCs, 16S rRNA and *mcrA* gene abundance were significantly higher than during the first spring frozen period in the 0–5 cm layer (Fig. 2). 16s rRNA gene copies and *mcrA* gene abundance increased by 81.9% and 215% respectively following spring FTCs. The expression of *mcrA* transcript was slightly higher during spring thawed period, although not significantly different from the frozen period prior to the FTCs. In the 5–10 cm layer, the abundance of the 16S rRNA and *mcrA* genes abundance remained constant.

3.4. Structures of bacterial and archaeal communities during FTCs

Illumina MiSeq sequencing of 16 composite soil samples produced a total of 558,409 raw sequence reads. After Ns were trimmed and lengths were split, 429,196 quality sequence reads were obtained and averages per sample were 26,824 reads. OTU clustering resulted in 9596 OTUs at a similarity level of 97%. The obtained bacterial and archaeal sequences were classified into 29 known phyla and mapped to 380 known genera. Archaea accounted for 3.33% to 4.52% of the total population, of which >70% were putative methanogens.

At the phylum level, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Proteobacteria* and *Firmicutes* were dominant groups in Sanjiang seasonal frozen marsh and together accounted for >95% of the total bacterial community, aside from unclassified bacteria (Fig. 3). Less abundant phyla (<0.5% of the average relative abundance) such as *Gemmatimonadetes*, *Planctomycetes*, *Chlorobi*, and *Nitrospirae* were also present in the study area during autumn and spring FTCs.

3.5. Methane cycling populations during FTCs

In seasonal frozen marshes, methane cycling is an important ecosystem process mediated by methanogens and methanotrophs, with methane release as the direct net result of methanogenesis and methane oxidation processes. We identified a total of 5356 sequences associated with known methanogens, which accounted for 1.25% of the total population. We found that acetoclastic methanogens (*Methanosarcinales*) and hydrogenotrophic methanogens (*Methanobacteriales*, *Methanocellales* and *Methanomicrobiales*) were all present in the Sanjiang seasonal frozen marsh during autumn and spring FTCs (Fig. 4). Acetoclastic methanogens were predominant, accounting for >90% of all methanogens.

A total of 452 sequences were associated with known methanotrophs. The relative abundance of methanotrophs was significantly lower than that of methanogens in each sample (Fig. 4). *Methylobacter* (11 OTUs) were the dominant genus belonging to the Type I methanotrophs. Type II methanotrophs *Beijerinckia*, *Methylobacterium*, and *Methylocystis* were also present.

Table 1
Physical and chemical characteristics of soils during autumn FTCs of 2014 and spring FTCs of 2015 in the Sanjiang seasonal frozen marsh (one-way ANOVA) (mean ± standard error (SE), n = 4).

Phase		Depth (cm)	Temperature (°C)	Water content (%)	pH	Dissolved organic carbon (DOC) (mg/kg)
Autumn	Frozen	0–5	−0.13	$41.94 \pm 0.83^{b,c}$	5.08 ± 0.03^{e}	544.63 ± 21.16^{b}
	Unfrozen	5–10	0.51	$38.56 \pm 0.66^{d,e}$	5.23 ± 0.01^{c}	362.39 ± 18.88^{d}
	Thawed	0–5	0.08	$40.56 \pm 0.76^{b,c,d}$	5.08 ± 0.01^{e}	706.96 ± 35.27^{a}
	Unfrozen	5–10	0.34	$42.06 \pm 0.62^{b,c}$	5.06 ± 0.01^{e}	360.08 ± 23.26^{d}
Spring	Frozen	0–5	−0.21	43.13 ± 0.82^{b}	5.41 ± 0.01^{a}	$423.40 \pm 24.22^{c,d}$
	Frozen	5–10	−0.21	37.94 ± 0.68^{e}	5.37 ± 0.01^{b}	381.14 ± 24.43^{d}
	Thawed	0–5	0.59	47.00 ± 1.49^{a}	5.14 ± 0.01^{d}	$475.78 \pm 34.34^{b,c}$
	Frozen	5–10	−0.16	$39.69 \pm 0.51^{c,d,e}$	5.21 ± 0.01^{c}	387.90 ± 25.22^{d}

Note: The same letter indicates no significant difference, and different letters indicate significant differences ($P < 0.05$).

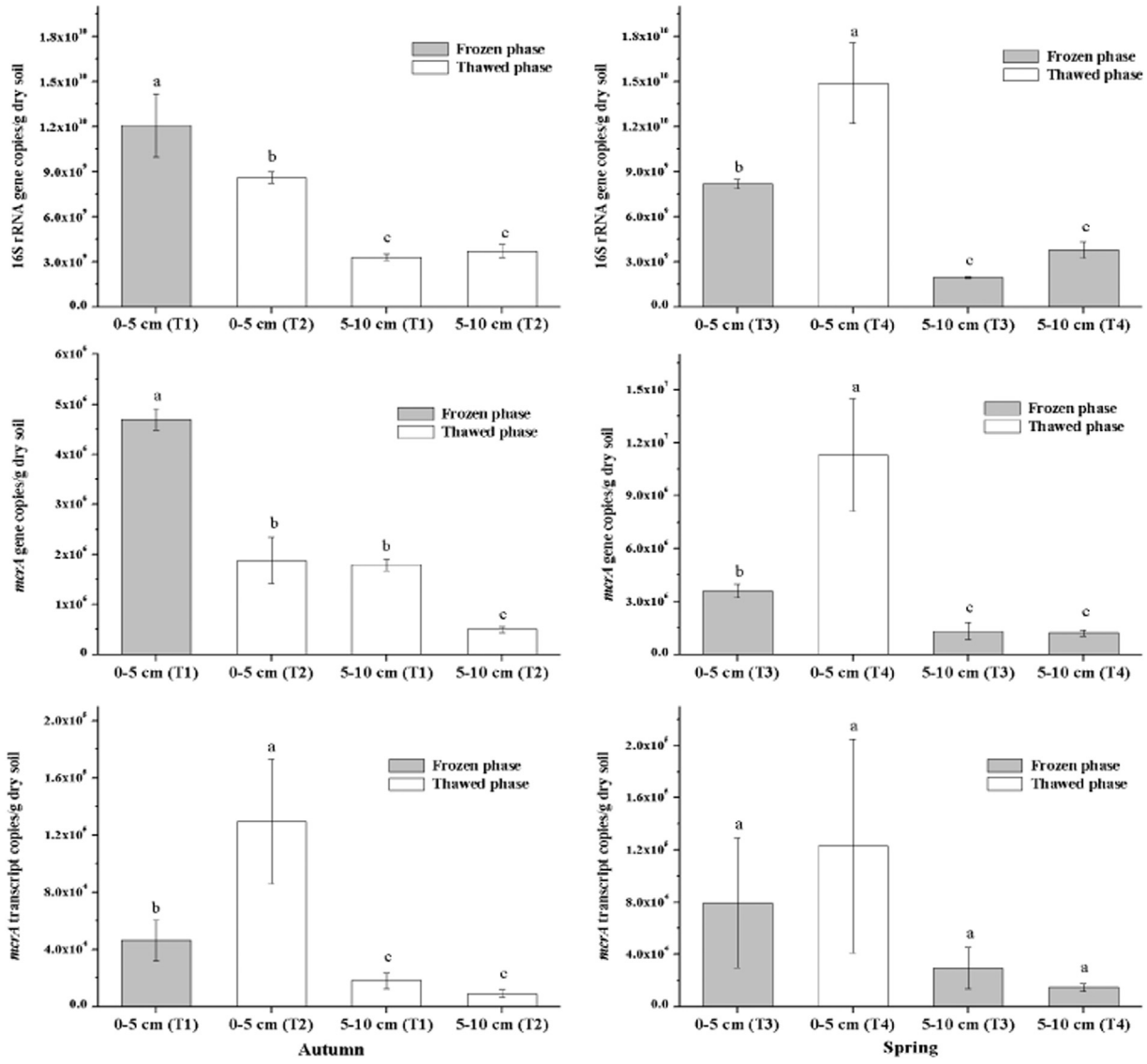


Fig. 2. Quantification of 16S rRNA gene, *mcrA* gene and its transcripts during autumn and spring FTCs in the Sanjiang seasonal frozen marsh (Duncan's multiple comparisons test). Note: T1: autumn frozen phase; T2: autumn thawed phase; T3: spring frozen phase; T4: spring thawed phase; the same letter indicates no significant difference, and different letters indicate significant differences ($P < 0.05$) (mean ± standard error (SE), n = 4).

3.6. Composition of structural changes in soil microbial community during FTCs

The soil microbial community maintained a consistent alpha diversity over the entire sampling period. There was no significant difference in species richness between frozen and thawed periods in either spring or autumn, or between the spring and autumn seasons overall (Fig. 5).

PCoA based on weighted UniFrac distances revealed the effect of FTCs on the beta-diversity of bacterial and archaeal communities (Fig. 6). The two dimensions together explained 63.66% of the observed variation. Microbial community composition between autumn frozen and thawed period differed significantly (Fig. 6, Table S2).

Between autumn frozen and thawed period, seven phyla, including 62 genera of soil microorganisms, exhibited significant changes ($P < 0.05$) (Fig. 7). At the phylum level, *Acidobacteria* and certain *Gammaproteobacteria* had higher abundance during autumn thawed period. However, *Firmicutes* and certain *Deltaproteobacteria* had higher relative abundances during frozen periods. Among *Firmicutes*, most genera, with the exception of *Planococcus*, demonstrated significant increases during autumn frozen periods ($P < 0.05$). Among *Deltaproteobacteria*, the abundance of *Bdellovibrionales*, *Desulfarculales*, *Myxococcales* and *Syntrophobacterales* increased in autumn frozen period. *Actinobacteria* maintained high relative abundance levels during FTCs. During spring FTCs, the composition of soil microorganisms did not change sharply (Fig. 7).

The two dimensions together explained 78.6% of changes in the soil microbial community structure (Table S3) during autumn FTCs. Redundancy analysis (RDA) indicated a significant relationship between bacterial community structure and DOC (Fig. 8). Furthermore, there were significant and strong correlations between the overall community composition and soil temperature. In addition, pH and water content determined 4.4% of shifts in bacterial composition.

4. Discussion

4.1. Effect of autumn and spring FTCs on bacterial and archaeal abundance

Our results demonstrated a significant decrease in the abundance of total bacteria and methanogens following autumn FTCs but an increase following spring FTCs. Previous work has shown that the effects of FTCs

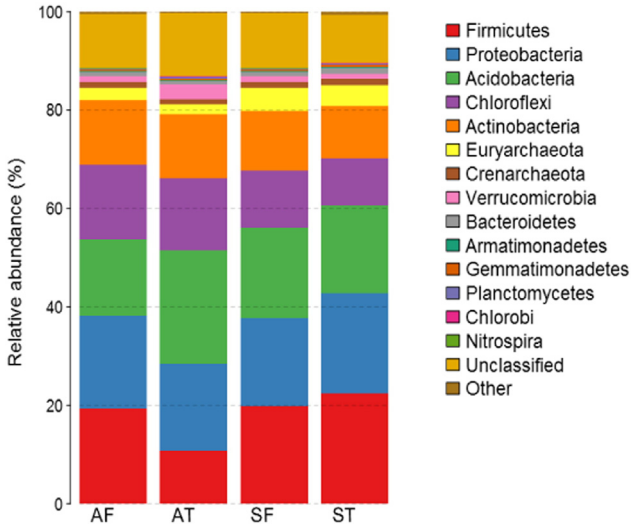


Fig. 3. Phylum composition distributions during different seasonal FTCs in the Sanjiang seasonal frozen marsh. Note: Other <0.1% of the average relative abundance; AF: autumn frozen; AT: autumn thawed; SF: spring frozen; ST: spring thawed (n = 4).

on soil microbial communities can range from subtle to detrimental (Feng et al., 2007; Henry, 2007; Walker et al., 2006). Autumn decrease can be explained by two possible reasons. One reason is that a larger microbial biomass may be present in the soil before autumn FTCs due to the transition from growing season to autumn FTCs. The other reason is that soil microorganisms may be unable to cope with FTCs stress and may die. Soil microorganisms which can withstand autumn FTCs may be resistant to winter low temperature condition. In terms of spring FTCs, increasing soil temperature and water content may lift low temperature and water activity restriction and increase the activity of soil microorganisms (Morozova and Wagner, 2007).

Changes in potentially active methanogens are different than those in total methanogens. The *mcrA* transcript expressed higher levels during autumn thawed period than during frozen period, indicating that methanogens may produce more methane during autumn thawed period. Soil microbial activity was higher during thawed period than frozen period (Sawicka et al., 2010). The expression of *mcrA* transcript slightly increased over the course of spring FTCs, indicating that a potential temporal lag of activity expression after soil microbes recover from freezing. The other studies found that soil bacteria exhibit delayed growth after a freeze-thaw event (Koponen and Baath, 2016). Our results confirmed the first hypothesis that autumn FTCs decreased the abundance of bacteria and methanogens, whereas spring FTCs increased the abundance of bacteria and methanogens.

4.2. Effect of seasonal FTCs on microbial community structure

Autumn FTCs did not change the alpha-diversity of soil microbes, but did change their beta-diversity. Furthermore, bacterial abundance remained nearly constant after autumn FTCs, implying that autumn FTCs select the microbial community, which then persists through spring FTCs. Psychrotolerant and dormant microorganisms may be important for buffering the composition and function of microbial communities in response to environmental changes (Makhalanyane et al., 2016). Neither the alpha-diversity nor beta-diversity of soil microbes was affected by spring FTCs.

In lines with some pervious studied (Haei et al., 2011; Mackelprang et al., 2011; Tveit et al., 2015), bacterial community composition underwent dramatic changes upon a freeze-thaw event. The community compositions of soil bacteria are similar between our study area and water logged permafrost in the Arctic (Hultman et al., 2015). Moreover, *Firmicutes* and some *Proteobacteria* showed the same changing trend with increasing temperature. However, the other studies had found FTCs had little impact on the microbial community composition using temperature gradient gel electrophoresis (TGGE) (Koponen et al., 2006), denaturing gradient gel electrophoresis (DGGE) (Yergeau and Kowalchuk, 2008) and terminal restriction fragment length polymorphism (T-RFLP) techniques (Mannisto et al., 2009). The inconsistent results were probably due to varying community composition of soil microbes including more psychrophilic and psychrotolerant bacteria in permanently cold environment before FTCs, or due to the sensitive of the experimental method.

Autumn FTCs can select and enrich dormant bacteria, strictly anaerobes and psychrotolerant bacteria by low temperature and anaerobic environment. During autumn FTCs, we found that *Firmicutes* and *Deltaproteobacteria* had relative higher abundances during autumn frozen periods. Some *Proteobacteria* and *Firmicutes* were acclimated to be active in sub-zero temperatures (Hultman et al., 2015). *Firmicutes* comprises spore-forming groups, such as *Clostridia*, which can withstand environmental disturbances (Paredes-Sabja et al., 2011). Among *Deltaproteobacteria*, *Bdellovibrionales*, *Desulfarculales*, *Myxococcales* and *Syntrophobacterales* were the main changing genera. Both *Desulfarculales* and *Syntrophobacterales* are strictly anaerobes.

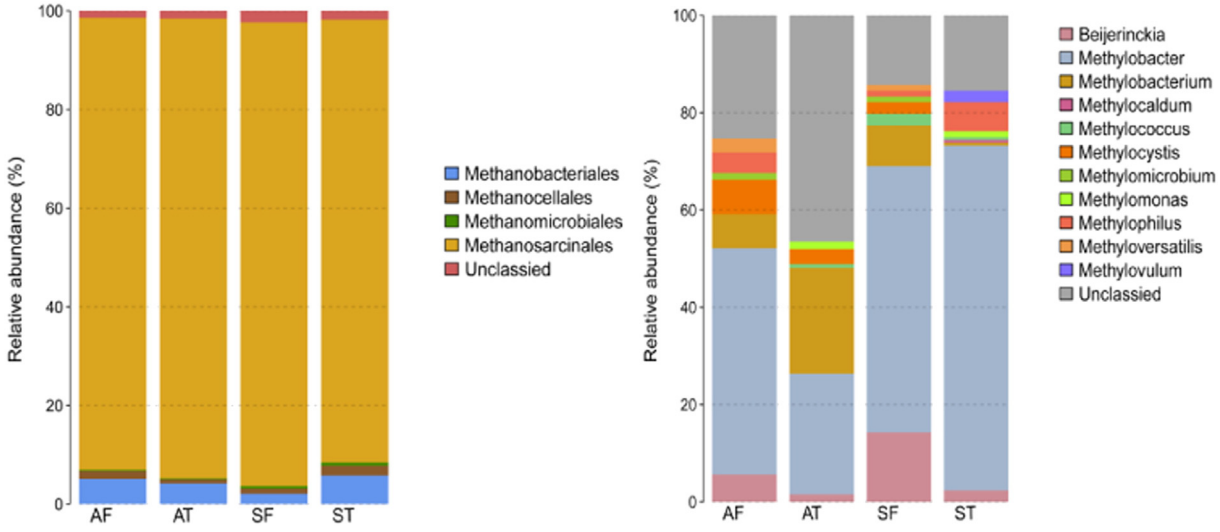


Fig. 4. Order composition and distribution of methanogens (left) and genus composition and distribution of methanotrophs (right) during different seasonal FTCs in the Sanjiang seasonal frozen marsh. Note: AF: autumn frozen; AT: autumn thawed; SF: spring frozen; ST: spring thawed (n = 4).

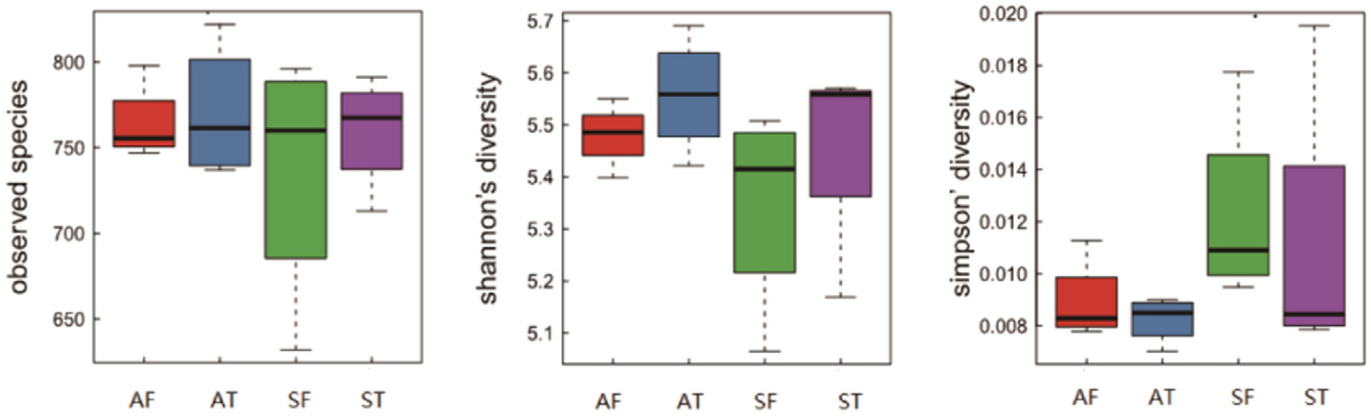


Fig. 5. Boxplot of alpha diversity indices during different seasonal FTCs (Kruskal-Wallis test). Note: AF: autumn frozen; AT: autumn thawed; SF: spring frozen; ST: spring thawed (n = 4).

Myxococcales can release myxospores and produce fruiting bodies in unfavorable environments. However, *Acidobacteria* showed higher abundance during autumn thawed period. In boreal cold and acid wetlands, *Acidobacteria* play an important role in degrading cellulose (Pankratov et al., 2011). This implied that soil microorganisms had stronger degrading function in autumn thawed period than autumn frozen period. Other FTCs studies also found higher microbial activity and larger gas emissions during thawed period than frozen period (Sawicka et al., 2010). Our results did not support the second hypothesis that multiple FTCs did not decrease the diversity of bacterial and archaeal communities in neither spring nor autumn.

4.3. The origins of CH_4 emission during spring thaw

FTCs and water log in our study area create stricter anaerobic conditions which is a prerequisite for methanogenesis. FTCs can increase anaerobic respiration in agricultural soils (Koponen et al., 2006). Additionally, our results showed that DOC concentrations were highest in 0–5 cm layer, which increased by 29.9% after the autumn FTCs (Table 1). This increase in DOC coincides with a decrease in bacterial abundance. Neither total bacterial abundance nor DOC changed in the 5–10 cm layer, which did not experience multiple diurnal FTCs. Therefore, our results suggest that autumn FTCs may facilitate the release of DOC through the lysis of soil microorganisms, which consistent with other studies (Haei et al., 2011). FTCs promote methane production by increasing anaerobic conditions and substrate availability for methanogens.

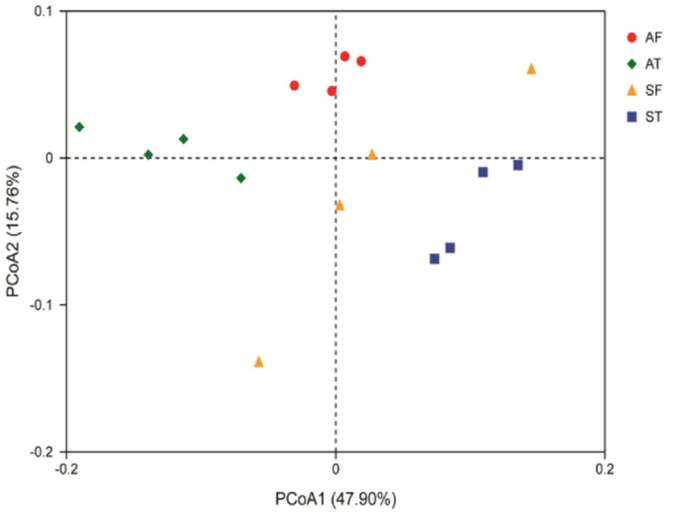


Fig. 6. Principal coordinates analysis (PCoA) of microbial community among different seasonal FTCs. The symbol shapes represent sampling periods and each point corresponds to an individual sample (AF: autumn frozen, AT: autumn thawed, SF: spring frozen, ST: spring thawed). Dissimilarities among communities were calculated based on weighted UniFrac distances.

Anaerobic fermentation was in favor of acetate accumulation (Duddleston et al., 2002). Acetate accumulation may the reason for soil acidification and dominant acetoclastic methanogens during autumn FTCs. DOC concentrations declined significantly from autumn thawed period to spring frozen period. The decline in DOC indicates that acetoclastic methanogens may utilize acetate and produce methane throughout autumn FTCs. The other study showed that microbial respiration remained at a high level in multiple diurnal FTCs (Larsen et al., 2002). Soil microorganisms maintain metabolic processes under freezing and subzero conditions (Hall et al., 2010; Tilston et al., 2010). Moreover, the relative abundance of methanotrophs was significantly lower than that of methanogens. Thus, less CH_4 may be oxidized by methanotrophs during autumn FTCs. Together, CH_4 comprising the surge most likely originated from cryogenic decomposition of soil organic matter by soil microorganisms during entire autumn FTCs, which lasted for approximately 72 days.

The emission period of CH_4 produced and accumulated in FTCs layer and the unfrozen subsoil fully coincided with the timing of the spring thaw during about four days. Koponen and Martikainen (2004) emphasized the roles of diffusion and advection barriers in the creation of gaseous emission bursts after thawing. Other research has shown that FTCs events trigger sudden, brief increases in microbial respiration (Sawicka et al., 2010; Schimel and Clein, 1996). In wetlands, nearly 50–90% methane is oxidized by methanotrophs before being released into the atmosphere (Le Mer and Roger, 2001). This indicates that the speed of soil thaw may determine CH_4 oxidation flux. Due to slow thaw, large amounts of methane stored in permafrost layers are oxidized to CO_2 (Schuur et al., 2009). However, the rapid bypass of the oxic zone reduces methane oxidation by methanotrophs. Overall, our results support our third hypothesis that spring CH_4 mainly comes from microbial production throughout autumn FTCs.

According to our RDA analysis, temperature and DOC were the primary environmental factors of regulating bacterial community composition during autumn FTCs. Soil temperature at Sanjiang seasonal frozen marsh fluctuated between −0.2 and +0.6 during FTCs (Fig. S2). Water content did not change significantly during autumn FTCs. Higher freezing temperature may not restrict free water which soil microorganisms can utilize. When moisture became available, soil microorganisms

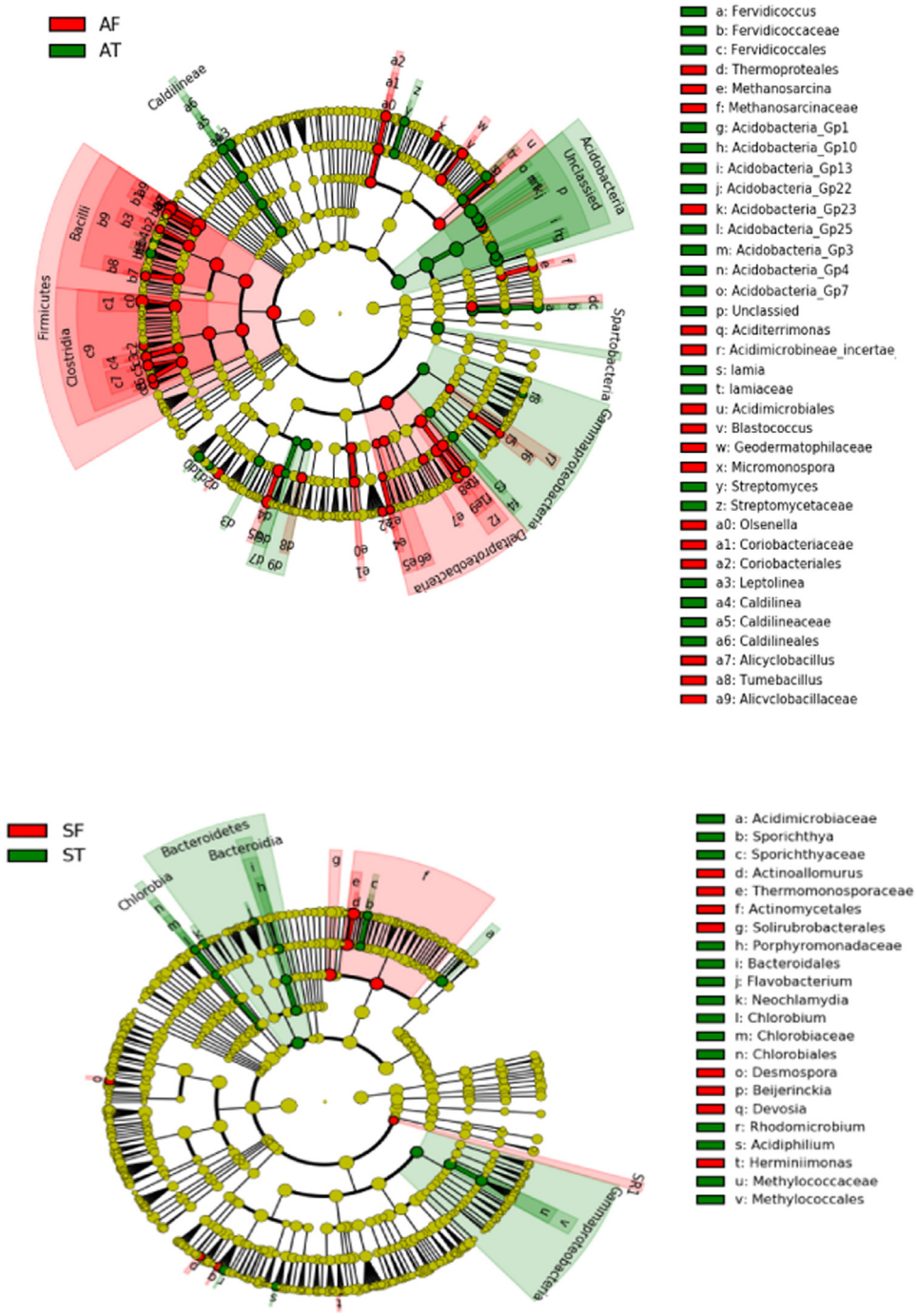


Fig. 7. A linear discriminant analysis effect size (LEfSe) method identifies the significantly different abundant taxa of bacteria and archaea between autumn and spring FTCs (AF: autumn frozen; AT: autumn thawed; SF: spring frozen; ST: spring thawed). Differences are represented in the color of the most abundant taxa (red indicate freeze, green indicate thaw, and yellow are non-significant). Each circle's diameter is proportional to the given taxon's relative abundance. Circles represent phylogenetic levels from domain to genus from the inside outwards.

responded more to fluctuations in temperature and substrate availability (Aanderud et al., 2013). This relatively small temperature amplitude during FTCs can promote more water-extracted organic carbon release and kill fewer soil microorganisms (J.Y. Wang et al., 2014). We observed that the abundances of active methanogens were higher during the thawed period, indicating larger potential CH_4 production. Various studies had reported that FTCs can promote substrates release by destroying soil aggregates and soil microorganisms (Koponen et al., 2006; Kreyling et al., 2008; Yergeau and Kowalchuk, 2008). Ongoing global climate change is predicted to increase the frequency of FTCs in cool-temperate and other high-latitude regions (IPCC, 2007). Greater numbers of FTCs induced by climate warming may lead to more CH_4 emissions in seasonal frozen marshes.

5. Conclusions

We analyzed the composition of soil bacterial and archaeal communities and the abundances of active methanogens in a seasonal frozen marsh of Northeast China during autumn and spring FTCs. The differing effect of autumn and spring FTCs on the populations of bacteria and archaea primarily depends on the community composition and abundance of soil microorganisms. Autumn FTCs promoted substrate

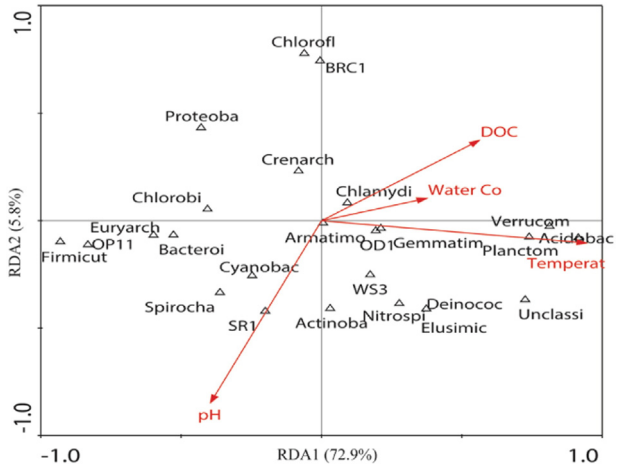


Fig. 8. Ordination triplot based on RDA (the model was significant at $P = 0.04$) of the relationships between soil microbial community structure (relative OTU abundances) and environmental variables during autumn FTCs. The red arrows and triangles represent environmental factors and soil microbial phyla, respectively.

release, and active methanogens had higher abundance during diurnal thaw period. Our results suggested that large CH_4 emissions in spring may mainly result from microbial production during entire autumn FTCs. Temperature and substrates were the main factors affecting the composition and quantities of soil microbial communities during autumn FTCs in seasonal frozen marsh. By promoting conditions conducive to methanogen activity, increased FTCs may lead to greater methane production in seasonal frozen marshes during cold seasons.

Acknowledgments

This research was financially supported by the National Key Research and Development Project (No. 2016YFA0602303), the Key Research Program of Frontier Sciences, CAS (No. QYZDJ-SSW-DQC013) and the National Natural Science Foundation of China (Nos. 41730643 and 41671105). We thank Fuxi Shi, Chao Gong, Wenwen Tan and Xinhou Zhang for their assistance at the field site. We also thank three anonymous reviewers for their constructive comments on the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.12.309.

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